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Targeted Disruption of the Catalytic Subunit of the DNA-PK Gene in Mice Confers Severe Combined Immunodeficiency and Radiosensitivity

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Summary

The DNA-dependent protein kinase is a mammalian protein complex composed of Ku70, Ku80, and DNA-PKcs subunits that has been implicated in DNA double-strand break repair and V(D)J recombination. Here, by gene targeting, we have constructed a mouse with a disruption in the kinase domain of DNA-PKcs, generating an animal model completely devoid of DNA-PK activity. Our results demonstrate that DNA-PK activity is required for coding but not for signal join formation in mice. Although our DNA-PKcs defective mice closely resemble Scid mice, they differ by having elevated numbers of $CD4^+CD8^+$ thymocytes. This suggests that the Scid mice may not represent a null phenotype and may retain some residual DNA-PKcs function.

Introduction

During lymphocyte differentiation, the variable regions of immunoglobulin (Ig) and T cell receptor (TCR) genes are assembled from their component variable (V), diversity (D), and joining (J) gene segments by means of a site-specific DNA recombination process referred to as V(D)J recombination (Tonegawa, 1993). This reaction is targeted by conserved recombination signal sequences (RSS) that flank each germline coding gene segment, and the process is initiated by the generation of precise DNA double-stranded breaks (dsb) between the elements to be assembled. Subsequent processing and ligation leads to the fusion of the four ends, generating two types of joins (for reviews see Lewis, 1994; Taccioli and Alt, 1995; Willerford et al., 1996; Ramsden et al., 1997). This multifactorial reaction is normally restricted to lymphoid cells, and the products of the recombination activating genes *Rag1* and *Rag2* (Schatz et al., 1989; Oettinger et al., 1990; Mombaerts et al., 1992; Shinkai et al., 1992) provide all the lymphoid-specific components required to initiate the reaction (for review see Ramsden et al., 1997).

Evidence that ubiquitously expressed proteins recruited from the dsb repair (dsbr) pathway are also required to complete the V(D)J recombination process first came from the characterization of Scid mice (Lieber et al., 1988; Malynn et al., 1988; Blackwell et al., 1989; Fullop and Phillips, 1990; Bosma and Carroll, 1991; Biedermann et al., 1991; Hendrickson et al., 1991) and later from the characterization of radiosensitive Chinese hamster ovary (CHO) cell lines defective in V(D)J recombination, which include *xrs-6*, XR-1, V-3, and irs-20 (Lieber et al., 1988; Taccioli et al., 1992, 1993, 1994a, 1994b; Pergola et al., 1993; Li et al., 1995; Jeggo, 1997; Lin et al., 1997).

The genes (*XRCC4*, *XRCC5*, and *XRCC7*) defined by complementation of the defects display by the mutant CHO cell lines have been cloned. The products of *XRCC5* and *-7* are two members of a protein complex, the DNA-dependent protein kinase (DNA-PK), a mammalian protein Ser/Thr kinase that must be bound to DNA in order to be activated. Biochemical analyses have established that this complex contains three subunits, Ku70 (*XRCC6*) and Ku80 (*XRCC5*), which are the binding components of this complex, and a 460 kDa catalytic subunit DNA-PKcs (*XRCC7*) (Getts and Stamato, 1994; Rathmell and Chu, 1994; Smider et al., 1994; Taccioli et al., 1994b; Boubnov et al., 1995; Jeggo et al., 1995; Anderson and Carter, 1996; Errami et al., 1996; Lees-Miller, 1996; Mizuta et al., 1996; Jeggo, 1997; Singleton et al., 1997). The role in vivo of Ku70 and Ku80 in V(D)J recombination has been recently confirmed by generation of deficient mice by gene targeting (Nussenzweig et al., 1996; Zhu et al., 1996a; Gu et al., 1997; Ouyang et al., 1997).

In contrast, a role in dsbr and V(D)J recombination for the *XRCC7* gene has been implicated based on a substantial body of indirect evidence (Blunt et al., 1995, 1996; Kirchgessner et al., 1995; Peterson et al., 1995; Lin et al., 1997; Peterson et al., 1997; Priestley et al., 1998).

The Scid mouse, which arose spontaneously, has served as the murine model for deficiency of DNA-PK activity. Characterization of the DNA-PKcs in Scid identified a nonsense mutation in the highly conserved C-terminal region of this gene (Blunt et al., 1996; Danska et al., 1996; Araki et al., 1997) localized downstream of the conserved Ser/Thr-phosphatidylinositol 3 kinase (PI3K) domain (Hartley et al., 1995; Poltoratsky et al., 1995; Araki et al., 1997). Although this mutation results in a large decrease in DNA-PKcs protein, residual DNA-PKcs protein can be detected at varying levels in Scid cell lines of different origin. Notably, the mutant *scid* DNA-PKcs protein retains the kinase domain, providing a structural basis for the possibility of residual DNA-PK activity in Scid (Kirchgessner et al., 1995; Peterson et al., 1995; Blunt et al., 1996; Danska et al., 1996; Araki et al., 1997).

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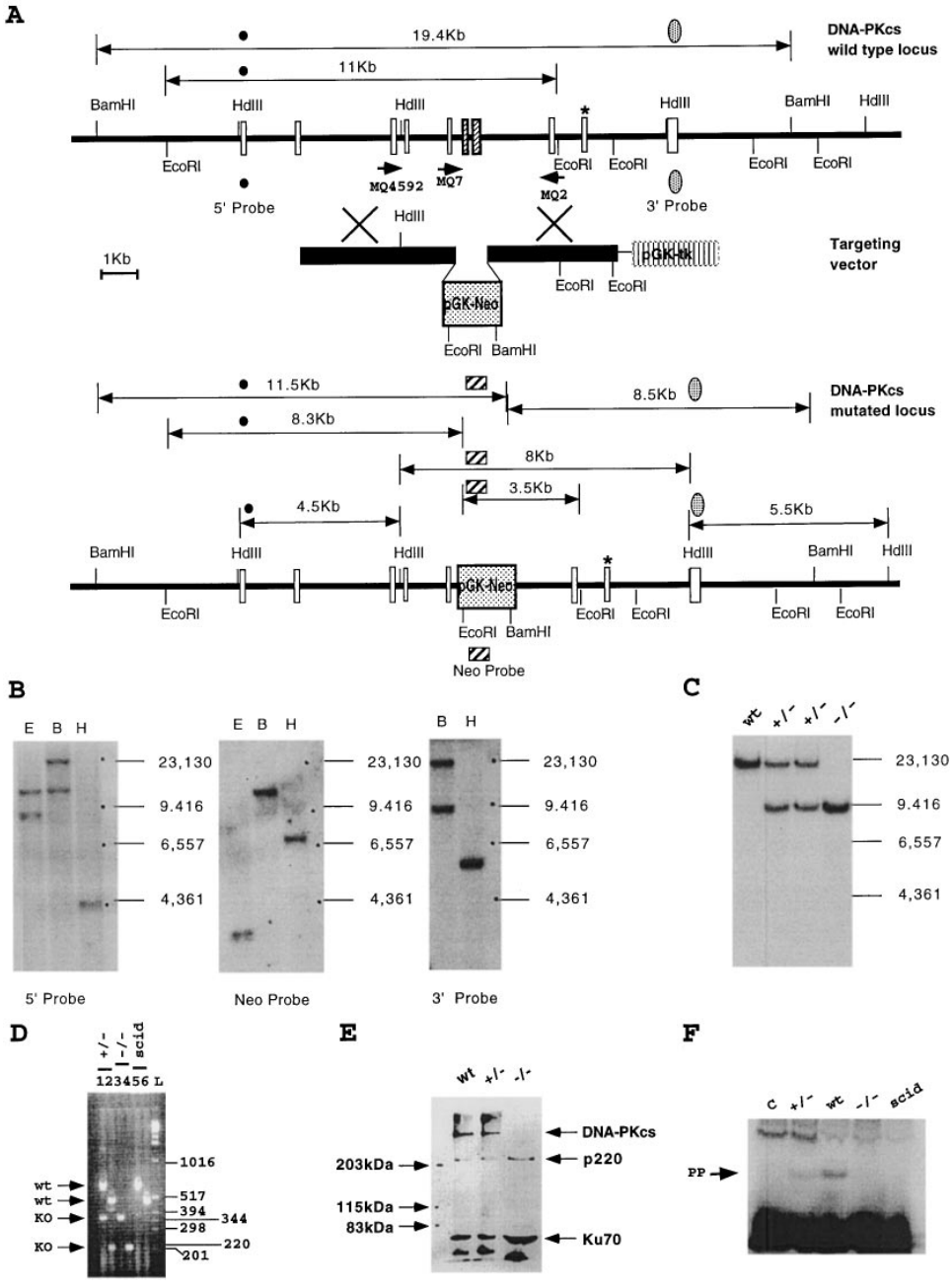

Figure 1. Inactivation of the Catalytic Domain of the DNA-PKcs Gene by Homologous Recombination

(A) Diagrammatic representation of the C-terminal portion of the DNA-PKcs locus (top), targeting construct (middle), and targeted allele (bottom) including relevant restriction sites, probes, and primers utilized. Exons are represented by boxes and arrows indicate primer positions. Shaded boxes represent exons containing key domains associated with catalytic activity and deleted by targeting. The asterisk represents the localization of the null mutation found in Scid mice.

(B) Southern blot analysis of ES cell DNA from clone BQN 142 following digestion with various restriction enzymes and hybridized with different probes derived from regions shown in (A). DNA size markers (λ phage digested with HindIII) are indicated in kilobases on the right side of the gel. E, EcoRI; B, BamHI; H, HindIII.

(C) Southern blot of BamHI-digested tail DNA from control (wt), heterozygous (+/−), and homozygous (−/−) DNA-PKcs mice. The DNA was hybridized with the 3′ probe to discriminate between the wt allele (19.4 kb) and the mutated one bearing the targeting vector (8.5 kb).

(D) RT–PCR from kidney RNA isolated from heterozygous (+/−), homozygous (−/−), and Scid animals. PCR products derived using the primer sets indicated are separated in ethidium-bromide (EtBr)-stained agarose gels. Lanes 1, 3, and 5 utilized primers MQ4592 and MQ2, and lanes 2, 4, 6 utilized primers MQ7 and MQ2. The location of the primers are shown above (A). Arrows indicated expected size of either wild-type (wt) or mutated (KO) PCR products. DNA λ ladder (GIBCO BRL) size markers are expressed in base pairs (lane L).

(E) Western blot analysis showing that DNA-PKcs protein is not expressed in DNA-PKcs-deficient animals. Whole-cell lysates prepared from fresh mouse thymocytes (100 μg) were separated in a 6% SDS-PAGE, transferred to nitrocellulose membrane, and probed with anti-DNA-

Genetic studies in Scid Arabian foals have correlated the presence of a null mutation upstream of the kinase domain of the DNA-PKcs gene with immunodeficiency. Significantly, the equine Scid model system differs from the murine model in being defective in both signal and coding join formation (Shin et al., 1997).

Here, by gene targeting, we have constructed a mouse with a disruption in the kinase domain of DNA-PKcs, generating an animal model devoid of DNA-PK activity. Our results demonstrate that DNA-PK activity is required for coding but not for signal join formation in mice and show that V(D)J recombination in the equine animal models differs from that in the mouse model. Although our DNA-PKcs-defective mice closely resemble Scid mice, there are significant differences, suggesting that Scid may indeed retain some level of residual DNA-PKcs function.

Results

Targeted Disruption of the DNA-PKcs Gene

To unravel the role of the DNA-PK activity in V(D)J recombination and, in turn, in lymphocyte differentiation, we generated mice containing a germline disruption of the *XRCC7* gene.

DNA-PKcs is an unusually large protein of approximately 460 kDa that is transcribed from a mRNA estimated to be around 13 kb in size (Hartley et al., 1995; Poltoratsky et al., 1995; Araki et al., 1997). Examination of the DNA-PKcs sequence reveals no significant homology to other known proteins, except in a 400 amino acid (aa) residue region in the carboxyl terminus. This region is highly related to the C-terminal catalytic domains of proteins falling into the PI3K superfamily (for review see Anderson and Carter, 1996; Lees-Miller, 1996; Jeggo, 1997), containing motifs which in conventional protein kinases play critical roles in catalysis. Outside of this region, no obvious motifs are evident, except for a leucine zipper domain near the middle of the DNA-PKcs sequence (Hartley et al., 1995; Poltoratsky et al., 1995; Araki et al., 1997).

To generate targeting constructs that inactivate the kinase domain by homologous recombination, genomic fragments containing sequences flanking this region were isolated using human partial cDNAs spanning the kinase domain as probes. A number of mouse genomic phage clones, covering nearly 25 kb of genomic sequences, were isolated. The partial genomic clones spanned about 3.5 kb of the C-terminal portion of the DNA-PKcs and include the Ser/Thr kinase domain and 3′ untranslated region. The genomic organization of the C-terminal portion of the DNA-PKcs gene (representing approximately 15% of the coding sequence of this enzyme) is outlined in Figure 1A.

The targeting vector was designed so that targeted integration by homologous recombination would yield a 1 kb deletion spanning two exons. The deleted exons contain motifs (amino acids 3860–3950; DXXXXN and DFG motifs) that are conserved between other Ser/Thr protein kinases and among species (Blunt et al., 1996; Shin et al., 1997) and are considered to play an important role in catalysis (see Figure 1A).

Two independently targeted embryonic stem (ES) cell clones carrying the disruption in the DNA-PKcs gene were further characterized utilizing three different restriction enzymes and two additional probes (5′ probe and *neo* probe described in Figure 1B). These results confirmed the predicted size bands and demonstrated the correct targeting of the *neo* gene into the DNA-PKcs locus and the absence of any additional ectopic integration (Figures 1B and 1C). One of these clones was injected into C57BL/6 blastocysts to generate chimeric mice. The mutant genotype was successfully transmitted through the germline after chimeras were crossed with Black Swiss females. No obvious defects were observed in the DNA-PKcs$^{+/-}$ heterozygotes that were crossed to generate DNA-PKcs$^{-/-}$ mice, which arose at Mendelian rates (Figure 1C). Adult homozygous mice were viable and fertile and were successfully bred in a modified barrier facility.

To confirm that the gene disruption produced an altered DNA-PKcs transcript, RNA was isolated from kidneys and analyzed by reverse transcription-polymerase chain reaction (RT–PCR). The results demonstrate that a fragment of approximately 300 bp is eliminated from the transcript derived from the disrupted allele (Figure 1D). The ablation of 90 aa generated in this targeting study spans the kinase domain and is upstream of the murine *scid* mutation (see Figure 1A). As a control, primers MNK3-MNK12, located close to the N-terminal end of the DNA-PKcs, were utilized for PCR, and an identical band of the expected size was generated with both alleles (data not shown). Sequence analysis of the RT–PCR product with primers MQ3 and MQ7 confirmed the absence of any coding *neo* sequences in the mature transcript. In addition, translation of the sequence of the RT–PCR product detected a protein truncation of 260 aa from the C-terminal end that confirmed the presence of a stop codon downstream of the frame shift created by the targeting event (data not shown).

To assess DNA-PKcs protein expression, we performed Western blot analysis on extracts from freshly isolated thymus from wild-type (wt), heterozygous, and DNA-PKcs$^{-/-}$ mice (Figure 1E). We have previously observed that thymus has higher levels of DNA-PKcs protein compared to spleen, bone marrow, or liver (data not shown). A 220 kDa protein that cross-reacts with certain DNA-PKcs antibodies has previously been reported by several groups and does not appear to represent a proteolytic cleavage product of DNA-PKcs (Figure

PKcs (18–2) and anti-Ku70 antibody as loading control. Cross-reactive material of approximate molecular weight 220 kDa is also indicated (220). Homozygous, (−/−); wild type, wt.

(F) Cell extracts prepared from DNA-PKcs$^{-/-}$ thymocytes lack DNA-PK activity. Assays were conducted in the presence of a p53 derived peptide (0.1 mM) and the phosphorylated product (PP) was resolved by polyacrylamide gel electrophoresis. As a control (C), the same amount of wt cell extracts was incubated without peptide and processed in parallel with the rest of the samples. Homozygous, (−/−); heterozygous, (+/−); wild type, (wt).

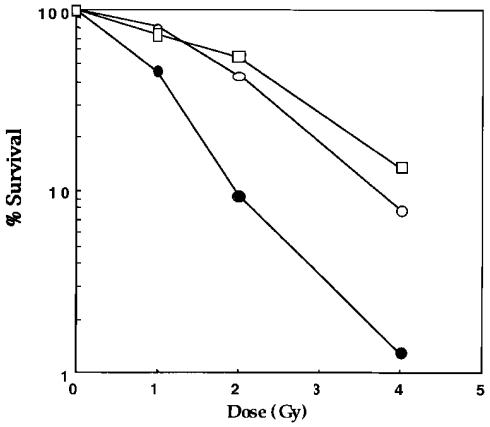

Figure 2. MEFs Established from DNA-PKcs Mice Are Radiosensitive

Survival of mouse embryonic fibroblasts (MEFs) established from homozygous DNA-PKcs-deficient mice (closed circle); heterozygous (open circles) and wt (open squares) MEFs following exposure to different doses of γ-irradiation.

1E) (Danska et al., 1996; Peterson et al., 1997; Priestley et al., 1998).

While DNA-PKcs protein can be detected in the thymus extracts from wt and heterozygous mice, no residual protein could be detected in the samples from our DNA-PKcs$^{-/-}$ animals (Figure 1E).

To extend the studies described above, DNA-PK activity has been investigated in the same extracts utilized for Western analysis. DNA binding proteins were first microfractionated using DNA double-strand cellulose and tested for the ability to phosphorylate a consensus DNA-PK recognition motif within a peptide derived from the N-terminal region of p53. The labeled product was resolved by polyacrylamide gel electrophoresis (indicated by an PP→ in Figure 1F). Whereas thymic extracts from wt control mice had considerable DNA-PK activity, thymic extracts derived from DNA-PKcs-deficient animals had no detectable activity. The lack of activity was also observed in liver and spleen extracts prepared from DNA-PKcs$^{-/-}$ animals (data not shown). We conclude that disruption of the DNA-PKcs gene inactivates the kinase activity of the DNA-PK complex. Significantly, DNA-PK activity from heterozygous animals was reproducibly nearly half of that observed in wt mice.

MEFs Established from DNA-PKcs Mice Are Radiosensitive

Mouse embryonic fibroblasts (MEFs) established from homozygous, heterozygous, and wt animals were examined for radiation sensitivity as described elsewhere (Priestley et al., 1998). MEFs from DNA-PKcs mice displayed the anticipated radiosensitivity when compared to heterozygous and wt controls (Figure 2). MEFs established from heterozygous mice appear slightly, but not significantly, more sensitive than the parental control.

Developmental Block of B and T Lymphocytes in DNA-PKcs-Deficient Mice

To asses the impact of loss of DNA-PK activity on V(D)J recombination, we examined 20 DNA-PKcs-deficient mice ranging in age from 8 days to 6 weeks for a number of parameters associated with lymphocyte differentiation.

First, we observed that the DNA-PKcs$^{-/-}$ thymi were disproportionately smaller, with 10- to 50-fold fewer cells than control wild-type (wt) or heterozygous (+/−) littermates. Spleen and lymph nodes were also smaller, with 5-fold fewer cells in the spleens of DNA-PKcs$^{-/-}$ mice relative to controls. Histologically, splenic white pulp nodules from two representative animals were significantly reduced and thymi lacked a distinct corticomedullary junction (data not shown).

To characterize further the immunological defect in DNA-PK$^{-/-}$ mice, cells from spleen, thymus, and bone marrow were analyzed by flow cytometry using monoclonal antibodies specific for cell surface markers. Consistent with the histological data, an early arrest in T and B cell development was found, as shown by the analysis of representative 2-week-old mice in Figure 3.

Development of T and B cells is an ordered process controlled by in-frame rearrangements and expression of antigen receptor genes. Rearrangements at the TCRβ locus start at an early stage of T cell development and are characterized by cells that lack expression of CD4 and CD8 (double negative [DN]) and the presence of CD25. Successful rearrangement of the TCRβ chain is required for the transition to CD4$^+$CD8$^+$CD25$^-$ (double positive [DP]) pre-T cell stage, in which TCRα chain is assembled. Expression of successfully rearranged TCRα chain allows developmental progression to CD4$^+$ or CD8$^+$ (single-positive [SP]) mature thymocytes harboring a complete TCRαβ complex on their surface that are ready to migrate to peripheral lymphoid organs (for review see Willerford et al., 1996).

Thymocytes from DNA-PKcs-deficient animals displayed an accumulation of pre-T cells at the CD44$^-$ CD25$^+$ stage, as shown in Figure 3. The early arrest in T cell differentiation is also confirmed by the absence of SP (Figure 3) and of TCRαβ$^+$ (data not shown) thymocytes when compared to either wt or heterozygous littermates. Further phenotypic analysis revealed a greater accumulation of DP cells in 90% of the DNA-PKcs-deficient animals analyzed compared to Scid. However, the percentage of DP cells was highest in 8-day-old mice (43%–45% of total thymocytes) and decreased with age. In addition, higher variability seemed also to increase with age. Mature T cell populations detectable by flow cytometry are normally present in the periphery of either wt or heterozygous mice but were not detected in 2-week-old DNA-PKcs-deficient animals by flow cytometry (Figure 3). In the spleen of 6-week-old homozygous mice, SP were detected at a frequency of less than 5% (data not shown) and cell populations expressing the myeloid marker CD11b (Mac-1) and the natural killer (NK) marker DX5 were increased compared to age-matched control mice (data not shown).

B cell maturation involves rearrangement of functional immunoglogulin heavy chain (IgHC), a process that is initiated in pro-B cells (B220^{lo}CD43$^+$) in the bone marrow. Successful assembly and expression of IgHC allows the cell to progress to a pre-B (B220$^+$CD43$^-$) cell stage where immunoglobulin light chain (IgLC) is assembled, a process accompanied by changes in the

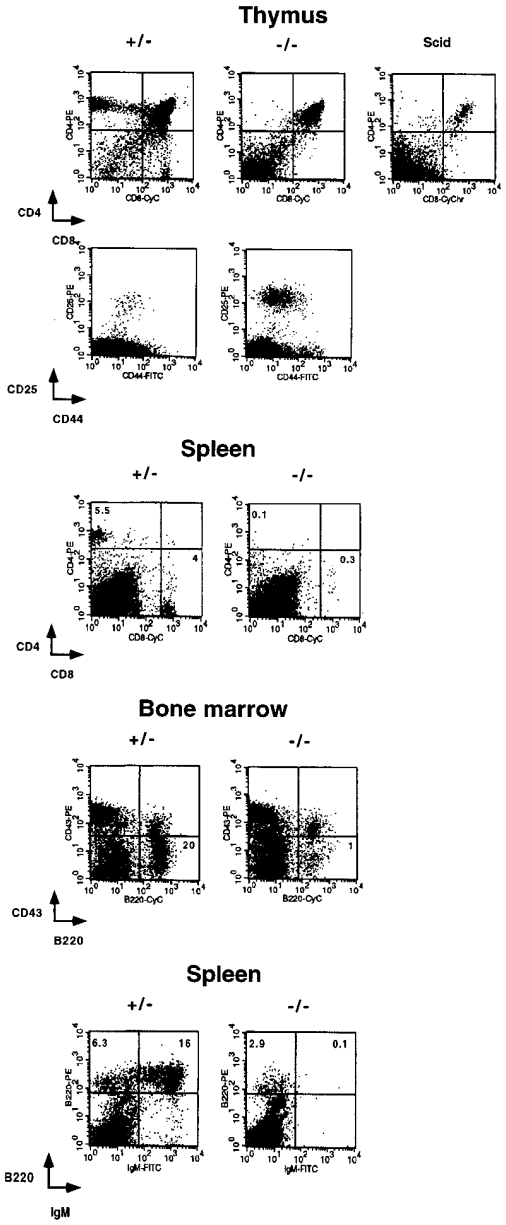

Figure 3. Lymphocytes from DNA-PKcs-Deficient Mice Are Blocked at an Early Stage of Development

Thymocytes (A), splenic (B), and bone marrow (C) lymphocytes isolated from 2-week-old DNA-PKcs-deficient mice and control littermates were analyzed by flow cytometry. Single-cell suspensions were prepared from the organs described and stained with fluorochrome-conjugated antibodies to CD4, CD8, CD3, TCR$\alpha\beta$, CD25, CD44, CD43, B220, and IgM. Triple staining CD4, CD8, and CD3 confirmed that DP are CD3$^-$.

expression of surface markers. Mature lymphocytes with a complete Ig receptor can migrate to the peripheral lymphoid organs (for review see Willerford et al., 1996).

Analysis of the B cell compartment of 2- (see Figure 3) and 6-week-old (data not shown) DNA-PKcs-deficient mice also showed an early arrest in the development at the B220$^+$CD43$^+$IgM$^-$ stage in the bone marrow and a lack of cells expressing surface IgM cells in the spleen. This apparent B cell block was confirmed by the absence of detectable serum IgM in 2-week-old DNA-PKcs ($-/-$) mice (data not shown).

Collectively, all these data demonstrate the requirement of DNA-PKcs for B and T cell development and resemble the phenotype described for Scid mice. However, the DNA-PKcs$^{-/-}$ mice here differ from Scid in having an elevated accumulation of DP thymocytes.

Abnormal Ig and TCR Recombination in DNA-PKcs$^{-/-}$ Mice

To determine whether the ablation of the kinase domain of the DNA-PKcs affects antigen receptor gene recombination, DNA from bone marrow, spleen, and thymus of 2- to 6-week-old animals were amplified with primers designed to specifically detect coding and signal join formation at Ig and TCR loci. As negative controls, DNA from Rag2$^{-/-}$ bone marrow, spleen or thymus, and tail were included in all PCR assays.

Using PCR analysis, the expected-size amplified products representing D-J_H4 and V_H7183-DJ_H4 coding join formation for IgHC and V_κ-J_κ2 for IgLC rearrangements are clearly seen in both wt or heterozygous animals but are severely reduced in DNA-PKcs$^{-/-}$, as shown in Figure 4A. Similar results were also obtained for D-J_H4 rearrangements using another primer, D_{Hall}, that recognizes a broad spectrum of D_H members (data not shown). Similarly, V-DJ_H4 rearrangements were analyzed employing primers that recognized other V_H family members like JH558, VQ52, or a degenerate V_{Hall} primer that recognizes most of the V_H region members, with similar results (data not shown). In addition, V_κ-J_κ2 rearrangements studies were extended to the spleen and comparable results to those obtained with bone marrow were observed (data not shown). Collectively, these data account for the absence of mature B cells and the undetectable level of IgM in the serum of the DNA-PKcs-deficient mice. These results were again similar to those found in Scid.

The analysis of TCR rearrangements also showed deficiencies. Only rare TCR V_β8-DJ_β2.6 coding joins were detected by PCR amplification of DNA from DNA-PKcs-deficient thymocytes (Figure 4B). In contrast, several preparations of DNA from heterozygous and wt littermates of different ages showed the characteristic pattern of bands for this type of T cell rearrangement. Similarly, coding joins indicative of V_α8-J_α50 (Figure 4B) and V_α8-J_α49 (data not shown) rearrangements were undetectable in DNA-PKcs-deficient animals but were observed in DNA from control mice. In addition, V_β8-DJ_β2.6 rearrangements in the spleen from 6-week-old DNA-PK-deficient mice were detected at much lower levels than in age-matched control animals (data not shown). The low level detected was consistent with the presence of a small number of mature T cells detectable by flow cytometry (data not shown). Coding join formation for D_δ2-J_δ1 was detected by PCR analysis, but V_δ1-DJ_δ1 was dramatically decreased and, indeed, undetectable in thymocytes of homozygous animals (Figures 5B and 5C).

To analyze signal join formation, primers derived from the sequences intervening between 3′ D_δ2 and the 5′

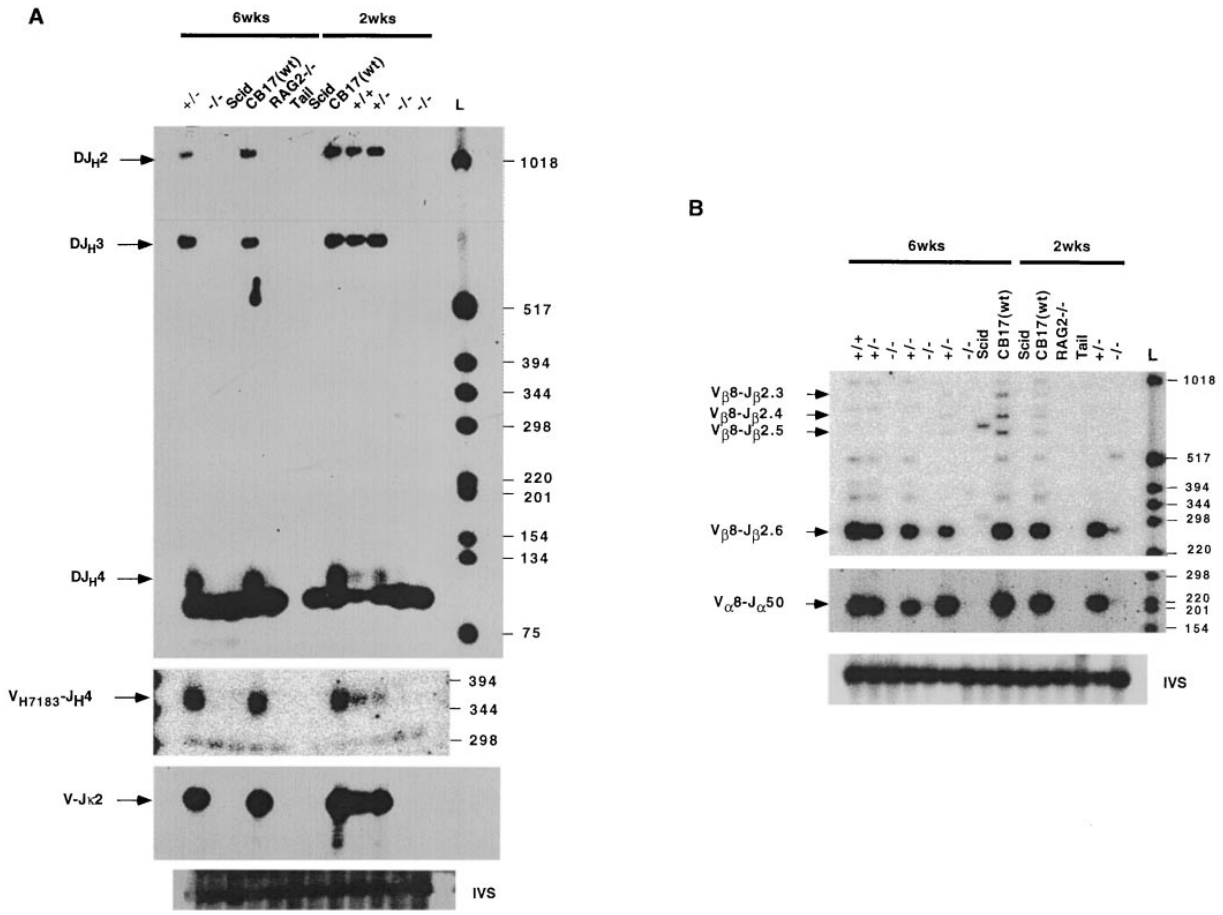

Figure 4. Coding Join Formation Is Dramatically Reduced in DNA-PKcs-Deficient Animals

(A) Ig PCR products derived from bone marrow DNA were analyzed for D-J_H4 and V_H7183-DJ_H for the IgHC and V-J_κ for the IgLC locus.

(B) PCR analysis of $V_\beta8$-$DJ_\beta2.6$ and $V_\alpha8$-$J_\alpha50$ rearrangements from thymocytes. Control DNA was amplified at the same time from tissue isolated from Scid (CB17 background), $Rag2^{-/-}$, and tail. An intervening sequence from the IgHC locus, not affected by Ig or TCR rearrangements, was amplified as control (IVS). Each lane represents an independently derived DNA sample from an individual mouse, and the results presented were reproducible in at least three independent experiments.

$J_\delta1$ RSS were utilized for PCR. These primers were able to amplify the stable circular DNA product that harbored the two juxtaposed RSS arising by excision after V(D)J rearrangement. Since precise signal join formation generates an ApaLI restriction site, digestion of the PCR product with this restriction enzyme allowed the precision of signal join formation to be assessed. The PCR products generated from DNA-PKcs$^{-/-}$ were present at comparable levels to those obtained from DNA prepared from wt or heterozygous animals (Figure 5D). In addition, at least 30% of the amplified RS join products from DNA-PKcs-deficient animals were sealed precisely and thus sensitive to ApaLI digestion. Further experiments confirmed the presence of signal join formation in nonstandard recombination $D_\delta1$-$D_\delta2$ product excised from thymocytes of DNA-PKcs-deficient animals at a comparable level with age-matched controls (data not shown). In addition to the signal join studies described above, ligation-mediated PCR (LMPCR) has also been employed to detect TCR $D_\delta2$-$J_\delta1$ intermediates. The results from three representative independent thymocyte DNA preparations confirm the data described above (Figure 5E).

To complement these studies, transient recombination assays were performed in MEFs as described previously (Taccioli et al., 1993). Rag1 and -2 expression vectors along with V(D)J recombination substrates for detection of either coding or RS joins were cotransfected into MEFs established from wt and DNA-PKcs$^{-/-}$ mice. Coding join formation was severely impaired in MEFs established from DNA-PKcs-deficient mice when compared to wt controls. In contrast, DNA-PKcs$^{-/-}$ MEFs performed RS joining nearly as efficiently as wild type; however, RS joins recovered from DNA-PKcs$^{-/-}$ MEFs were imprecise in nearly 50% of the events (data not shown).

In summary, mutation of the catalytic domain of the DNA-PKcs gene exerts a dramatic impairment in coding but not in signal join formation in vivo. These features are similar to those observed with murine Scid.

Discussion

In this study, we show that ablation of the catalytic domain of DNA-PKcs in mice results in a block of T and B cell differentiation with an accumulation of early

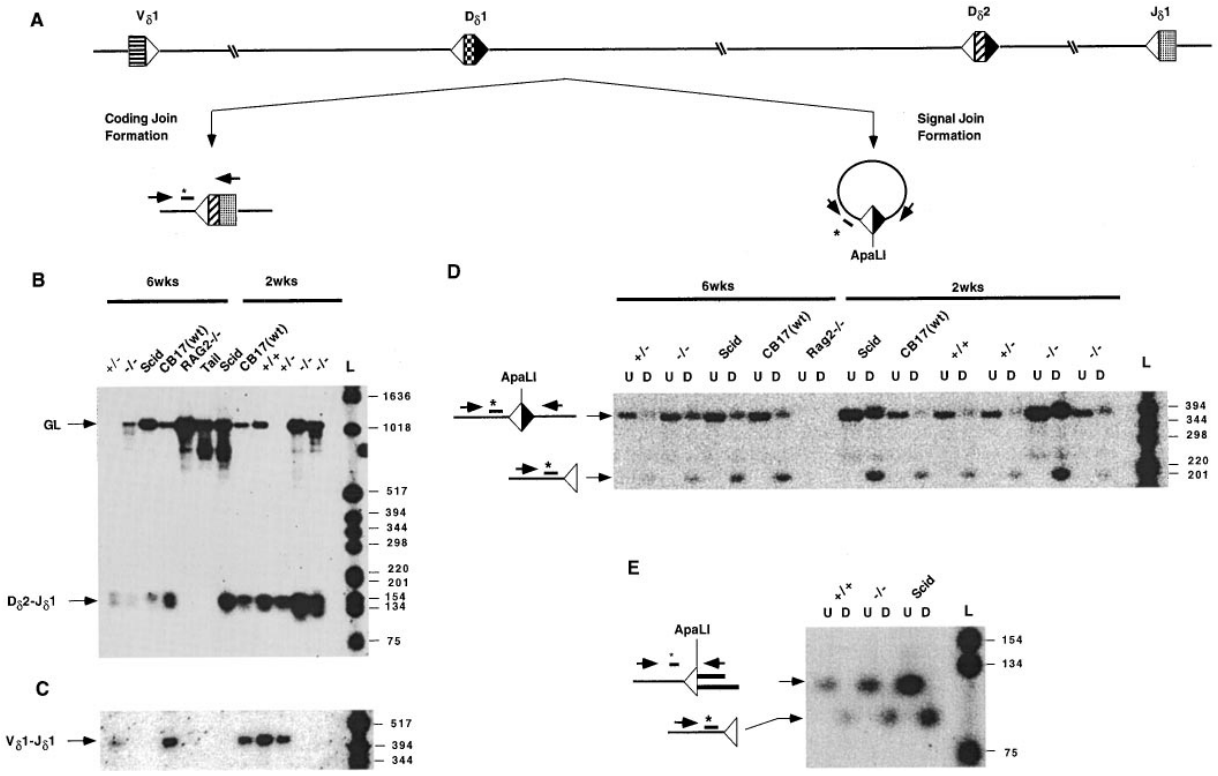

Figure 5. Signal Join Formation Is Mildly Affected in DNA-PKcs-Deficient Mice

TCRδ rearrangements were analyzed by PCR and LMPCR from DNA samples isolated from thymocytes of DNA-PKcs-deficient animals and control littermates at indicated ages.

(A) Schematic representation of the TCRδ locus. Boxes, coding segments; triangles, RSS. Thick arrows indicate PCR primers and lines with asterisks refer to internal oligonucleotides probes employed to hybridize PCR products.

(B and C) Detection of coding join formation by PCR. PCR products derived from thymus DNA were analyzed for $D_\delta2$-$J_\delta1$ (B) and $V_\delta1$-$DJ_\delta1$ (C) rearrangements for the TCRδ locus. Thin arrows indicate the expected size for the indicated rearrangement; GL, germ line. DNA λ ladder (GIBCO BRL) size markers are shown on the left side of the blots and sizes are expressed in base pairs (L).

(D) Detection of circular excised $D_\delta2$-$J_\delta1$ RS join by PCR. Fidelity of the RS join formation was evaluated by digestion with restriction enzyme ApaLI. D, digested; U, undigested.

(E) LMPCR detection of RS ends at the 5′ end of $D_\delta2$ segment. Precision of the RS join was evaluated as described in (D). Schematic representation of intermediates is indicated on the right side of the gel. The RS, represented by an open triangle, is shown as the cleavage product prior to ligation of double-strand primers (thick lines). An intact RS end creates an ApaLI restriction site upon ligation.

lymphocyte precursors, thus rendering a severe combined immunodeficiency phenotype. Associated with this defect, DNA-PKcs$^{-/-}$ animals exhibit severely impaired formation of coding joins in both T and B cells, providing evidence for the involvement in vivo of this gene product in V(D)J recombination. In contrast, signal join formation occurs at close to normal frequencies. Blunt and 5′ phosphoryated signal ends (Roth et al., 1993; Schlissel et al., 1993) are detected in thymocytes of DNA-PKcs$^{-/-}$ animals by LMPCR, similar to that observed with normal animals. Taken together, these results demonstrate that loss of DNA-PK activity exerts a differential impact on coding versus signal join formation.

This immunodeficient/radiosensitive phenotype resembles that of the Scid mouse and supports the evidence that the causal mutation in Scid is a defect in DNA-PKcs. The mutation in the DNA-PKcs-deficient mouse generated in our study results in a 260 aa truncation and loss of the entire kinase domain. With available techniques, we cannot detect any residual protein or kinase activity. In contrast, the mutation in the Scid mouse causes an 83 aa truncation resulting in loss of the highly conserved C-terminal region but retention of the kinase domain (Blunt et al., 1996; Danska et al., 1996; Araki et al., 1997). Moreover, residual protein can be detected in murine Scid cells, the level of which varies between cell types (Blunt et al., 1995; Kirchgessner et al., 1995; Peterson et al., 1995; Danska et al., 1996). Although residual kinase activity has not been detected in scid cells, limitations in the sensitivity of the assay preclude an assessment of whether the truncated protein retains residual kinase activity. Consequently, it has been difficult to exclude the possibility that some residual kinase activity might affect the Scid phenotype. The mutation in the equine Scid animals produces a stop codon in the C-terminal region of the DNA-PKcs transcript and, thus, a protein truncated by 967 aa. There is no residual protein or detectable kinase activity. Curiously, the equine Scid animals are defective in both signal and coding join formation, whereas the DNA-PKcs-deficient mouse, like murine Scid, only lacks coding join formation (Lieber et al., 1988; Blackwell et al., 1989; Pergola et al., 1993; Taccioli et al., 1992, 1994a;

Lin et al., 1997; Shin et al., 1997). We conclude, therefore, that signal join formation does not require DNA-PK activity in mice. We cannot exclude the possibility that DNA-PKcs may play some role in signal join formation separate from its function as a protein kinase, since our mice were constructed to lack specifically the kinase domain, and we cannot detect any residual protein. However, our interpretation is further supported by results reported in an accompanying paper by Gao et al. (1998 [this issue of *Immunity*]) in which a different target mutation located in the 5′ region of this gene also impacts only upon coding join formation.

The fact that the truncation generated in our mice produces dramatic reduction in protein expression is in line with the analysis of other DNA-PKcs-defective cell lines. Molecular characterization of DNA-PK-deficient mutants, including the hamster cell lines V-3 and irs-20 and Scid cells from murine and equine origin, identified mutations in the DNA-PKcs within the C-terminal 1.3 kb region, in some cases outside of the conserved PI3K domain. However, in all cases this is accompanied by decreased protein levels, suggesting that an intact C-terminal region is required for protein stability (Blunt et al., 1996; Danska et al., 1996; Araki et al., 1997; Shin et al., 1997; Priestley et al., 1998).

A distinctive feature found in DNA-PKcs-defective animals compared to murine Scid is a reproducible increase in the DP population of the thymus. However, we detect only low numbers (less than 5%) of SP in the periphery of older DNA-PKcs$^{-/-}$ animals, a feature also observed in Scid. The peripheral SP cells could represent a small population that apparently has bypassed the block to coding join formation; they cannot be attributed to a leaky phenotype resulting from residual kinase activity nor to a revertant phenotype, both explanations proposed for Scid (Petrini et al., 1990). Significantly, the presence of mature lymphocytes in Scid is dependent on housing conditions (Schuler, 1990; Bosma and Carroll, 1991). Although the DNA-PKcs knockout mice have been maintained in a modified barrier facility, more restricted housing conditions were utilized for the Rag2$^{-/-}$ and Scid mice raised in a defined flora environment at Taconic.

It is striking that the DNA-PKcs$^{-/-}$ mice here, which are devoid of DNA-PK activity, have 40%–45% DP thymocytes, whereas Scid mice have a barely detectable DP population in thymus. These results provide evidence that the Scid mouse may not represent a DNA-PKcs null phenotype and may have some residual DNA-PK function. Curiously, a murine transgenic line, Sra5–1, was recently generated by the fortuitous integration of several copies of a fragment of a yeast transgene into the N-terminal end of the DNA-PKcs gene. This mouse also accumulates an elevated number of DP thymocytes, but it is difficult to substantiate a causal relationship since the authors failed to provide conclusive evidence that the site of integration is unique (Jhappan et al., 1997).

A possible explanation for the difference between the DNA-PKcs$^{-/-}$ and Scid mice is that Scid mice do indeed retain some residual kinase activity, and if this supposition is correct, it raises the surprising observation that the magnitude of the DP population is inversely related to the residual kinase activity. A working model to explain the accumulation of DP in DNA-PKcs-deficient animals is that the lack of DNA-PK activity might allow thymic differentiation of DN to DP stage, but it does not allow for proliferation of DP since TCRβ rearrangement has not taken place. It is tempting to speculate that one function of DNA-PK activity might be to inhibit thymic differentiation of DN to DP stage in the absence of productive TCRβ rearrangement. Thus, promotion to the DP stage may require a competition of opposing regulators: a positive signal through a functional pre-TCR receptor that needs to overcome the negative regulation exerted by activation of DNA-PK. Such a control may serve to prevent thymocytes harboring double-stranded breaks from undergoing T cell differentiation until productive TCRβ rearrangement has taken place.

Elevated levels of the DP population in thymus are also observed in Ku70$^{-/-}$ mice, but in this case they are associated with "leaky" T cell development and normal thymus architecture (Gu et al., 1997; Ouyang et al., 1997). Ku80$^{-/-}$ mice also have a small accumulation of DP (less than 20%). While this may be explained by a different mechanism, it is also possible that these mice have a very low level of kinase activity, since DNA-PKcs is intact and potentially functional. It has recently been reported that DNA-PKcs can be activated, at least to some extent, by a Ku-independent pathway (Anderson and Carter, 1996; Yaneva et al., 1997; Hammarsten and Chu, 1998).

It is also worth noting that sublethal γ-irradiation of immunodeficient animals, including Scid (Danska et al., 1994; Murphy et al., 1994; Bogue et al., 1996; Livak et al., 1996; Zhu et al., 1996b), Ku80$^{-/-}$ (Nussenzweig et al., 1997), and Rag2$^{-/-}$ (Zuniga-Pflucker et al., 1994; Guidos et al., 1995; Jiang et al., 1996), with different doses results in an elevated DP population. Depending on the radiation dose, the extent of artificially created double-stranded breaks might exceed the physiological scenario and activate alternative signals that could bypass normal regulatory checkpoints. In physiological situations, antigen receptor rearrangement probably involves the generation of only a limited numbers of double-stranded breaks, and recruitment of the DNA-PK complex to those sites is necessary to repair them by a Ku-dependent pathway. The absence of DNA-PK activity may result not only in the abnormal accumulation of those double-stranded breaks, but also in the deregulation of other pathways involved in T cell maturation.

Experimental Procedures

Targeted Disruption of DNA-PKcs and Generation of Mice Lacking DNA-PK Activity

The replacement targeting vector BQN was constructed using genomic fragment isolated from a strain 129 library (Stratagene) cloned into the plasmid pLNTK as described elsewhere (Chen and Alt, 1994).

Embryonic stem cells (J1 ES cells) were grown on embryonic fibroblast feeder cells, electroporated in the presence of 40 μg of PvuI-linearized targeting vector DNA, and selected on feeder cells with active G418 at 150 μg/ml and 2 μM gancyclovir. Homologous recombinants were identified by Southern blotting of BamHI-digested DNA using the 3′ probe (see Figure 1C) and arose at a frequency of 1/150. The positive ES cell clone BQN 142 was injected into C57BL/6 blastocysts to generate chimeric mice.

All transgenic mice were housed with littermates in autoclaved cages with microisolator lids, and autoclaved food, water, and bedding were manipulated in a hood. Rag2$^{-/-}$, Scid(CB17), CB17, and Black Swiss mice were purchased from Taconic (Germantown, NY) and maintained/bred at Boston University Medical Center Animal Care Facility. Except for Black Swiss, other animals were originally maintained by the vendor in defined flora conditions and then bred in house under more stringent conditions than our transgenic animals.

Southern Blot Analysis for Identification of Wild-Type and Mutant Alleles

Genomic DNA isolated from ES cells was digested with different restriction enzymes (New England Biolabs), separated by electrophoresis through a 1% agarose gel, and transferred to nylon membrane (Zetaprobe-Bio Rad). Probes utilized have been described in Figure 1A and were ^{32}P-labeled by random primer protocol (Boehringer Mannheim).

RT–PCR Analysis

Total RNA was isolated from kidney for RT–PCR analysis using Trizol reagent (GIBCO BRL). Reverse transcription was performed with oligo-dT (Boehringer Mannheim) using 2 μg of total RNA and Superscript II (GIBCO BRL). PCR was performed using 1 μl of cDNA, 160 ng of each primer, and high fidelity polymerase (Boehringer Mannheim) following conditions provided by the manufacturer.

PCR products generated after 30 cycles (annealing temperature 60°C) were subcloned in PCR 2.2 (Invitrogen) vector and sequenced from dsDNA using the Sequenase kit (Amersham Life Sciences).

Oligodeoxinucleotides that flanked the exons ablated were MQ2, 5′-GTGAGCAGACCTGCACAAGAG; MQ7, 5′-AGACTGGCTGATGAA AGTGTC; and MQ4592, 5′-GACTTACCGTGTCGTGCCAA. Control oligodeoxinucleotides for N-terminal amplification were MNK3, 5′-CTGGTAAACATGATCCTCAG and MNK12, 5′-ACCTGCCTTCTGGA CAACTAT.

Cell Preparation and Flow Cytometry Analysis

Single-cell suspensions from lymphoid organs of 8-day- to 6-week-old mutant and littermate control mice were prepared for staining and analyzed on a Becton-Dickinson FACScan with Cell Quest software (Becton Dickinson, San Jose, CA) following standard procedures.

Bone marrow cells were harvested from femurs by syringe lavage, and cells from thymus and spleen were prepared with frosted slides. Dead cells were gated out by forward and side scatter properties.

The following antibodies (PharMingen) conjugated with fluorescein isothiocyanate (FITC), phycoerythrin (PE), biotin (bio), or CyChrome (CyC) were used: anti-Pan NK-PE (clone DX5), anti-CD25-PE (clone 3C7), anti-CD3e-FITC (clone 145–2C11), anti-TCRβ-PE (clone H57–597), anti-B220-PE (FITC, CyC) (clone RA3–6B2), anti-CD44-FITC (clone IM7), anti-CD43-FITC (PE) (clone S7), anti-CD8α-CyC (clone 53–6.7), and anti-CD4-PE (clone RM4–5). The anti-mouse IgM (μ chain)-FITC was purchased from Sigma (F-9259).

Isolation and Growth of MEFs

MEFs were prepared from day 13.5 embryos derived from crosses between DNA-PKcs$^{+/-}$ according to the National Institutes of Health 3T3 protocol.

DNA Preparation and PCR Analysis of V(D)J Recombination Intermediates and Products

DNA was prepared for PCR as described by Schlissel et al. (1991). Reactions (20 μl) contained 2–8 μl template (approximately 10,000–40,000 genomes, which correspond to 50–200 ng of DNA), 160 ng of each primer, 1 U Taq polymerase (Perkin Elmer), and buffer provided by the manufacturer containing 1.5 mM $MgCl_2$. Amplification (30 cycles) was performed at an annealing temperature of 65°C.

The reaction was analyzed in 2%–2.5% agarose gel and was alkaline transferred to a nylon membrane (Zetaprobe-Bio Rad).

Oligo probes were labeled with T4 polynucleotide kinase (New England Biolabs) and γ-^{32}P (New England Nuclear), and DNA fragments were labeled by random primer extension (Boehringer Mannheim) with DNA polymerase I (Klenow fragment) (New England Biolabs) and α-^{32}P (New England Nuclear).

For analysis of Ig coding joins, several sets of primer were utilized. To evaluate D-J_H rearrangement, the pair included the degenerate primer DHL or D_{Hall} and J_H4 (Schlissel et al., 1991; Guidos et al., 1995; Zhu et al., 1996a); for V-DJ_H rearrangement, the J_H4 was combined with V_H7183 (Zhu et al., 1996a), V_HJ558L (Ouyang et al., 1997), VQ52 (Schlissel et al., 1991), or V_{Hall} (Guidos et al., 1995). A J_H4-specific probe (DR218) was utilized for the detection of Ig coding join PCR products as described elsewhere (Zhu et al., 1996a). Evaluation of light chain was performed as described before with $J_κ2$ and $V_κ$deg primers (Schlissel and Baltimore, 1991).

TCR coding joins $V_β8$-$DJ_β2.6$ and $D_δ2$-$J_δ1$ were detected as described by Zhu et al. (1996a), and $V_δ1$-$DJ_δ1$ joins were detected by employing the primers P5 (Carroll et al., 1993b) and $V_δ1$ (Carroll and Bosma, 1991). The product was probed with oligo DR53 that is within the $J_δ1$ region (Zhu et al., 1996a). $V_α8$-$J_α50$ TCR coding joins were analyzed as described elsewhere (Zhu et al., 1996a).

Signal join formation for $D_δ2$-$J_δ1$ rearrangements were amplified by circular PCR using primers P2 and P6 and probed with P6-int oligo as described previously. Similarly, to detect the $D_δ1$-$D_δ2$ excised product, primers P3 and P4 were employed and the product was probed with DR2 (Carroll et al., 1993a, 1993b; Zhu et al., 1996a).

An intervening sequence from the heavy chain locus that is not affected by Ig rearrangement was amplified as a control for PCR reaction using 5′-IVS and 3′-IVS primers (Nussenzweig et al., 1996; Ouyang et al., 1997) and probed with a genomic fragment spanning the J_H region of the murine IgM.

LMPCR Analysis

$D_δ2$ signal join ends were detected by LMPCR as described elsewhere (Roth et al., 1993). Primers DR19 and DR20 were preannealed, and this adapter ligated to thymus DNA allows the recreation of a restriction site for ApaLI when ligated to intact ends. PCR has been performed as described above employing primers DR20 and DR6 at 60°C. PCR product has been digested with ApaLI to determine the fraction of intact end when compared with undigested PCR products. The reaction was analyzed in 2.5% agarose gel and probed with oligo DR2 as described above.

Western Blot Analysis

Tissue extracts (100 μg) were resolved in a 6% SDS-PAGE loading buffer and transferred to nitrocellulose membranes. The DNA-PKcs antibodies utilized were 18–2 (Carter et al., 1990) and DNA-PKcs-3M, a rabbit antibody raised against the N terminus of mouse DNA-PKcs (Priestley et al., 1998). The Ku70 monoclonal antibody utilized was N3H10 (Taccioli et al., 1994b). Filters were developed using an enhanced chemiluminescence (ECL) kit (Amersham).

Protein Extract Preparation and Determination of DNA-PK Activity

Whole-cell extract from thymi and liver were homogenized as previously described (Finnie et al., 1995). The extracts (100 μg of each) were micropurified by pulldown on 5 mg of dsDNA-cellulose beads and washed twice in 50 mM Z′ 0.05 buffer. Assays were performed with a final concentration of 0.1 mM peptide and 0.1 mM ATP (containing 50 μCi of γ-^{32}P/mMol ATP) and incubated for 10 min at 30°C. Samples were resolved on 18.5% polyacrylamide gels using the Tris/tricine buffer system. The gels were dried and quantitation of incorporation of label into the peptide was measured by phosphorimager (phosphorimager and ImageQuant by Molecular Dynamics).

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